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T_{1D}-weighted ihMT imaging – Part II. Investigating the long and short T_{1D} components correlation with myelin content. Comparison with R₁ and the macromolecular proton fraction (MPF).

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ABSTRACT (word count: 239 words)

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2
3 **Purpose:** To investigate the long and short T_{1D} components correlation with myelin
4 content using ihMT high-pass and bandpass T_{1D} -filters and to compare ihMT, R_1 , and
5
6 the macromolecular proton fraction (MPF) for myelin specific imaging.
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10
11 **Methods:** 3D ihMTRAGE sequences with increasing switching times (Δt) were used to
12 derive ihMT high-pass T_{1D} -filters with increasing T_{1D} cutoff values and an ihMT
13 bandpass T_{1D} -filter for components in the 100 μs to 1 ms range. 3D SPGR-qMT
14 protocols were used to derive R_1 and MPF maps. The specificity of R_1 , MPF and ihMT
15
16 T_{1D} -filters was evaluated by comparison to two histological reference techniques for
17
18 myelin imaging.
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28 **Results:** The higher contribution of long T_{1D} s as compared to the short components as
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30 Δt got longer led to an increase in the specificity to myelination. In contrast, focusing
31 on the signal originating from a narrow range of short T_{1D} s (< 1 ms) as isolated by the
32 bandpass T_{1D} -filter led to lower specificity. In addition, the significantly lower r^2
33 correlation coefficient of the bandpass T_{1D} -filter suggests that the origin of short T_{1D}
34 components is mostly associated with non-myelin protons. Also, the important
35 contribution of short T_{1D} s to the estimated MPF, explains its low specificity to
36 myelination as compared to the ihMT high-pass T_{1D} -filters.
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49 **Conclusion:** Long T_{1D} components imaging by means of ihMT high-pass T_{1D} -filters is
50 proposed as an MRI biomarker for myelin content. Future studies should enable the
51 investigation of the sensitivity of ihMT T_{1D} -filters for demyelinating processes.
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4 **Keywords:** ihMT T_{1D} -filtering, long T_{1D} components, short T_{1D} components, myelin
5
6 specificity, myelin microscopy imaging
7

8
9 **Running title:** Investigating the long and short T_{1D} components correlation with myelin
10
11 content. Comparison with R_1 and the macromolecular proton fraction (MPF).
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15 **Body text word count: 5833 words**
16

17 18 INTRODUCTION

19
20
21 Myelination and demyelination mechanisms in the central nervous system (CNS) are
22
23 a hallmark of aging and pathology. High specificity and sensitivity imaging tools are
24
25 essential for an early detection of the variations in myelin content. The versatility of
26
27 MRI has endowed the neuroscience community with numerous non-invasive MR
28
29 markers sensitive to a variety of biophysical mechanisms and tissue microstructural
30
31 features. In particular, the myelin sheath, composed of 40% of water and 60% of dry
32
33 mass containing a high proportion of lipids (1), can be characterized by quantitative
34
35 MRI measures probing both the water and the macromolecular components (2–6).
36
37 While the issue of sensitivity has been addressed in multiple studies, the specificity to
38
39 myelination of various quantitative and semi-quantitative MR metrics remains an open
40
41 question.
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51 The macromolecular proton fraction (MPF) (7) is a quantitative metric derived from the
52
53 binary spin-bath model for MT (8,9), exhibiting a higher sensitivity to pathologically
54
55 induced variations than the classical MT ratio according to preclinical (10–12) and
56
57 clinical Multiple Sclerosis (MS) (13) studies. Standard methodology for MPF
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2
3 measurement involves the acquisition of multiple MT-weighted images sampling a
4
5 large number of offset frequencies and powers with regards to the preparation pulse
6
7 in spoiled gradient echo (SPGR) sequences. However, the latest techniques for
8
9 quantitative MT (qMT) allow the estimation of 3D MPF maps in a relatively short
10
11 acquisition time (single-point qMT) (13–17).
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16
17 The longitudinal relaxation rate (R_1) provides the basis for the most common contrast
18
19 used in clinical routine. Besides the high dependence of in vivo R_1 quantification on
20
21 the employed measurement technique (18), additional controversies are linked to the
22
23 dominant factor influencing the R_1 contrast. While some publications show a clear
24
25 relationship between iron deposition and R_1 (19,20), other studies suggest a greater
26
27 and more significant contribution from macromolecules (21,22). Nonetheless, R_1 is
28
29 shown to be a good predictor of the macromolecular tissue volume of the in vivo brain
30
31 throughout the whole lifespan (23) and allows differentiation between cortical layers
32
33 (24). In addition to macromolecules (25) and paramagnetic ions (26), R_1 is also
34
35 sensitive to tissue hydration (27) and protein content (28).
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44 Inhomogeneous magnetization transfer (ihMT) (29) is an MRI technique, weighted by
45
46 T_{1D} , the dipolar order relaxation time (30), an endogenous source of contrast driven by
47
48 slow molecular dynamics and tissue microstructure (31,32). The sensitivity of ihMT to
49
50 myelinated tissues (33–37) and myeloarchitectonic mapping (38) was validated in
51
52 studies on healthy subjects, and for the characterization of myelin pathology in MS
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54 patients (39,40). A previous study comparing ihMT with histology (41) on genetically
55
56 modified plp-GFP (proteolipid protein - Green Fluorescence Protein) mice highlighted
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1
2
3 the impact of distinct T_{1D} -filtering strategies on the sensitivity and the specificity of ihMT
4
5 to healthy myelinated tissues. As shown in Part I, high RF power and low duty-cycle
6
7 ihMT preparations are sensitive to at least two T_{1D} components. While high-pass T_{1D} -
8
9 filters can be used to adjust the signal contribution of long T_{1D} components (> 1 ms) to
10
11 the total ihMT signal, sensitivity to the short component is achieved by linear
12
13 combinations of data with different switching times, e.g., to produce an ihMT bandpass
14
15 T_{1D} -filter in the 100 μ s to 1 ms range. These filtering strategies inform on studies of
16
17 microstructural correlates in healthy myelinated tissues using the corresponding ihMT
18
19 images.

20
21
22 The aim of this work was thus to evaluate the specificity of ihMT high-pass and
23
24 bandpass T_{1D} -filters to myelination and compare it to that of MPF and R_1 . In addition
25
26 to ihMTR, ihMTsat metrics compensated for R_1 relaxation effects and B_1^+
27
28 inhomogeneity effects arising from the excitation pulses were computed. The
29
30 specificity to myelination was evaluated based on the estimated intercept and slope
31
32 values (41) from linear regressions between the MR metrics and myelin quantifications
33
34 using two microscopy measurements: plp-GFP fluorescence intensity signal and the
35
36 LFB (Luxol Fast Blue) optical density.

37 38 39 40 41 42 43 44 45 46 47 48 49 **METHODS**

50 51 52 *Animal experiments*

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55 Animal studies were conducted in agreement with the French guidelines for animal
56
57 care from the French Department of Agriculture (Animal Rights Division), the directive
58
59
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1
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3 2010/63/EU of the European Parliament and of the Council of 22 September 2010 and
4
5
6 approved by our institutional committee on Ethics in animal research (Comité d’Ethique
7
8
9 de Marseille n°14, project authorization APAFIS#1747-2015062215062372v6).

10
11 Sixteen C57Bl/6J control mice were scanned in vivo at 10 weeks of age. Among them,
12
13
14 6 genetically modified mice expressing the green fluorescent protein (GFP) under the
15
16
17 control of the *P₀* promoter which drives the expression of a major myelin component
18
19
20 in the CNS – the proteolipid protein (PLP) (42) were sacrificed after the MR scans and
21
22
23 analyzed by microscopy.

24
25 During scans under isoflurane anesthesia (1.5%, constant flow, 300 mL/min; Univentor
26
27
28 400 anesthesia unit, Zejtun, Malta), animal respiratory rate and temperature were
29
30
31 monitored (SA Instruments, Stony Brook, NY, USA). Respiration was maintained at
32
33
34 100 ± 10 breaths-per-minute using minor adjustments to the isoflurane concentration.
35
36
37 Temperature was maintained at 37.5 ± 0.5 °C using a heating blanket connected to a
38
39
40 water bath system.

41 42 ***Quantitative MR protocol and image processing***

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44
45 MR experiments were performed on a preclinical 7T scanner (Bruker PharmaScan,
46
47
48 Ettlingen, Germany), using a 72-mm body volume coil and a four-channel phased array
49
50
51 receive-only MRI CryoProbe (Bruker, Ettlingen, Germany) for RF transmission and RF
52
53
54 reception, respectively.
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4 After positioning in the magnet isocenter, a field map-based shimming (acquisition time
5
6 1 min 22 s) was performed to optimize B_0 field homogeneity over the entire mouse
7
8 brain.
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10
11 Structural T_2 -weighted (T_2w) images were acquired using a 2D multi-slice RARE
12
13 sequence (RARE factor = 8, TE/TR = 32/5030 ms, matrix size 256x256, in-plane voxel
14
15 size 78x78 μm^2 , slice thickness 300 μm , 49 slices covering the whole brain, acquisition
16
17 time TA = 5 mins 22 s). B_1^+ mapping was performed with the Actual Flip Angle imaging
18
19 (AFI) method (43,44) (TE/TR₁/TR₂ = 2/15/60 ms, matrix size 48x48x44, 400x400x750
20
21 μm^3 , FA = 60°, TA = 1 min 59 s) on the whole mouse brain.
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26
27

28 *R₁ maps*

29
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31 Variable Flip Angle VFA-SPGR sequences (TE/TR = 2.2/30.0 ms, TA = 9 mins 30 s)
32
33 for R_1 quantification were acquired using FAs of 6°, 10°, and 25°. Data were fit to the
34
35 Ernst equation (45) to estimate voxel-wise the R_1 values corrected for B_1^+ deviations.
36
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38
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40 *Single-point MPF maps*

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42
43 An MT-prepared SPGR sequence (TE/TR = 2.2/30.0 ms, TA = 3 mins 10 s) was
44
45 performed using a 10.25 ms off-resonance gaussian pulse for saturation with an offset
46
47 frequency of 6 kHz and a flip angle of 600° (corresponding $B_{1\text{peak}} = 9.1 \mu\text{T}$ and $B_{1\text{rms}} =$
48
49 5.0 μT) (17). According to the single-point strategy, the two-pool model for MT with
50
51 optimal constrained parameters (17) was used to estimate MPF maps with B_1^+
52
53 correction. The VFA-SPGR acquisition at FA = 10° was used as a reference image.
54
55
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57
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59 *lhMTR maps*

T_{1D} -weighted ihMT images were acquired with the sequence presented in Part 1 and the protocol described in Part I. In brief, slab-selective 3D low duty-cycle (35,46) ihMTRAGE (47) sequences were acquired with different dual-frequency offset switching times (Δt) (Saturation: 15 bursts of $N_p = 8$ Hann-shaped pulses, pulse duration $p_w = 0.5$ ms, $B_{1\text{peak}} = 42.4$ μT , offset frequency $\Delta f = 10$ kHz, burst repetition time BTR = 60 ms, total saturation time $\tau = 900$ ms, corresponding root-mean-squared saturation power calculated over the total saturation time $B_{1\text{RMS}}^{\text{SAT}} = 6.7$ μT and RF duty-cycle DC = 6.7%. Readout: TE/TR = 2.1/2200.0 ms, matrix size 192x192x8, voxel size 100x100x750 μm^3 , cartesian centric-out in the RAGE partition). IhMT images were calculated from the difference between the single and the dual-frequency offset MT images. The latter were obtained either with cosine-modulated pulses (dual-frequency switching time virtually corresponding to $\Delta t = 0.0$ ms and referenced hereafter as $\Delta t_{0.0}$), either with frequency-alternating pulses and $\Delta t = 0.8$ ms, and $\Delta t = 3.2$ ms (referenced hereafter as $\Delta t_{0.8}$ and $\Delta t_{3.2}$, respectively). Based upon the results of Part I, the selected Δt values led to ihMT high-pass T_{1D} -filters with increasing T_{1D} cutoff values. In addition, an ihMT bandpass T_{1D} -filter, isolating components within the 100 μs to 1 ms range, was obtained by subtracting ihMT images derived from the $\Delta t_{0.0}$ and $\Delta t_{0.8}$ configurations ($\Delta t_{0.0} - \Delta t_{0.8}$). For each configuration, a reference image with zero power saturation, MT_0 , was also acquired. IhMTR maps were calculated:

$$ihMTR = \frac{(MT^+ + MT^-) - (MT^\pm + MT^\mp)}{MT_0} \text{ equation 1}$$

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2
3 For SNR consideration, a total number of 6, 12, and 18 repetitions of MT^+ and MT^\pm
4
5 images were acquired for the $\Delta t_{0.0}$, $\Delta t_{0.8}$, and $\Delta t_{3.2}$ respectively, leading to acquisition
6
7 times of 8 mins 40 s, 11 mins 20 s, and 22 mins.
8
9

10 11 *IhMTsat maps*

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15 IhMT metrics corrected for R_1 relaxation and B_1^+ inhomogeneity effects on the readout
16
17 excitation pulses (ihMTsat) were derived based on a strategy initially proposed for MT
18
19 imaging (48), and more recently customized to the ihMTRAGE framework (38).
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21

22
23 Equations (A10) from Munsch et al. (38) were used to derive the longitudinal
24
25 magnetization at the first RAGE segment, taking into account all the events in the
26
27 sequence (i.e., saturation and free precession periods, and readout pulses). The
28
29 attenuation factor (δ), described under the hypothesis of a single instantaneous
30
31 saturation (6.4 ms burst), was estimated using the Brent's root finding method for both
32
33 single- and dual- offset saturations to derive ihMTsat.
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40 Python-based wrappers are made available: 1) for R_1 and MPF maps calculation at:
41
42 https://github.com/loustelle/SP_qMT_proc (hash cc210c2); 2) for ihMTsat maps
43
44 calculation at: https://github.com/loustelle/ihmt_proc (hash 34bd8b3).
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48 A diagram summarizing the acquired raw images and the processing steps required to
49
50 build the R_1 , MPF, ihMTR and ihMTsat metrics is provided in Figure 1.
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54 *MR images quantification*

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4 3D-templates of ihMTR, ihMTsat, MPF and R_1 were constructed and registered in the
5
6 same reference as the T_2w templates. A comprehensive description of the template
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8 construction from the individual images of the 16 mice, and the segmentation of
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10 Regions-of-Interest (ROIs) is provided in the appendix of Part I.
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13 *ROI-wise analyses (MRI vs. microscopy)*

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18 ROI-wise analyses on each of the 6 plp-GFP mice at 3 bregma levels (+ 0.7 mm, -
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20 1.0 mm and - 3.2 mm from bregma), corresponding to the microscopy slices, were
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22 performed for comparison between MR and microscopy metrics. ROI-based
23
24 quantification was performed from the averaged values in seven structures (mCC –
25
26 medial corpus callosum, INT – internal capsules, OPT – optical tract, TH – thalamus,
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28 CP – caudoputamen, CTX- cerebral cortex, and HIP – hippocampal region) for all MR
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30 and microscopy metrics.
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35 *Voxel-wise analyses (MPF vs. ihMT T_{1D} -filters)*

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40 A brain mask was generated from a hard thresholding procedure based on the MPF
41
42 template image, and applied to all co-registered templates (ihMTR, ihMTsat, MPF and
43
44 T_2w). In order to eliminate the CSF contribution in view of whole-brain voxel-wise
45
46 analyses, a 3-classes k-means clustering was performed using Atropos (49) from
47
48 ANTs based on the skull-stripped T_2w template image. Voxels were then retrieved for
49
50 each common slice of the ihMTR, ihMTsat and MPF templates. Analyses between
51
52 MPF and ihMT metrics were done on the voxel-wise averaged metrics of the 16 mice.
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59 *Microscopy images quantification*

1
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3 After in vivo MR experiments, the 6 plp-GFP mice were sacrificed (ketamine 150 mg/kg
4 with xylazine 15 mg/kg) and intracardially perfused with a fixative of (4%
5 paraformaldehyde). Mouse brains were then extracted, post-fixed for 2 h in 4%
6 paraformaldehyde and cryopreserved overnight in 20% sucrose. Frozen brains were
7 sectioned into 20 μ m thick histological slices (Cryostat Leica CM305S) in the rostro-
8 caudal direction at 3 different levels (+ 0.7 mm, - 1.0 mm and - 3.2 mm from bregma)
9 for plp-GFP fluorescence and LFB optical density analyses. ROIs were manually
10 placed on the microscopy images by two different observers (J.B. and M.C.) in the
11 seven brain structures chosen for the ROI-wise MRI quantification, using a mouse
12 brain atlas as reference.
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30 *Plp-GFP fluorescence*

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33 Six mouse brains were used for the plp-GFP fluorescence analysis. For each level,
34 three slices were acquired using a Zeiss fluorescence microscope with a 5x objective
35 and a fixed exposure time. Background noise was measured in the ventricles and
36 subtracted. Analyses were performed using the ImageJ software (National Institutes of
37 Health, Bethesda, MD) and the mean value in each of the seven structures was
38 computed. The resulting signal was normalized, taking as a reference the signal in the
39 internal capsules.
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51 *LFB optical density*

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55 Three mouse brains were used for the LFB staining analysis. Brain sections were post-
56 fixed in methanol for 2 mins at -20°C, then rinsed in ethanol/acetic acid (96/4) for 5
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3 mins. Sections were then stained in LFB (Sigma-Aldrich) 0.1% diluted in ethanol/acetic
4
5 acid during 3 h at 60°C. After a short rinse in ethanol 96%, sections were decolorized
6
7 in lithium carbonate 0.05% for 1 min and rinsed in ethanol 70%. Finally, sections were
8
9 dehydrated through increasing ethanol gradients (from 70% to 100%) and mounted in
10
11 Eukitt (VWR). Pictures were taken with a Zeiss Axiolmager microscope with a 5x
12
13 objective and optical density was measured using ImageJ, as described by
14
15 Khodanovich et al. (12). Briefly, the mean intensity of the red channel was measured
16
17 in RGB images as a quantity characterizing the complementary blue channel
18
19 saturation. Background signal was also measured in the ventricle, and LFB optical
20
21 density was calculated as follows: $(1 - \text{red mean intensity} / \text{background intensity})$.
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30 Signals were normalized to the signal of the internal capsules.

31 32 ***Analyses and Statistics***

33 34 *MRI vs. microscopy*

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39 Mean and standard deviation values in the seven averaged ROIs from the three
40
41 bregma levels of the 6 plp-GFP mice were used to evaluate the specificity of each MR
42
43 metric and the sensitivity of ihMTR and ihMTsat configurations. The normality of data
44
45 and that of residuals in regression analyses was assessed in JMP (v.16.0.0; SAS
46
47 Institute, Cary, NC) using the Shapiro-Wilk test. Pearson's correlations and linear
48
49 regressions were performed for a total of ten response variables (ihMT high-pass and
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51 bandpass T_{1D} -filters, MPF and R_1) and two predictor variables (normalized plp-GFP
52
53 and LFB signal intensities).
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Comparisons between the linear regression analyses

A one-way analysis of covariance (ANCOVA) with interaction in JMP was used to test for significance in differences between pairs of slopes and intercepts. Steiger's modification of Dunn and Clark's z approach (50) and the Zou's confidence interval (51) from the *cocor* package (52) for overlapping correlations based on dependent groups in R (v.1.4.1106; RStudio, PBC, Boston, MA, US) was used to test for significance in differences between pairs of correlation coefficients.

Two types of comparison analyses were performed. First, by considering the MR metrics as the common variable, we investigated whether the MR metrics behave significantly different when associated with plp-GFP or LFB signals. Second, by considering one of the microscopy metrics as the common variable, we investigated whether there is an MR metric that associates significantly better with each of the two microscopy references.

Specificity assessment

The specificity to myelin of all MR metrics (41) when referenced to either plp-GFP or LFB measurements was assessed as the fraction of MRI signal associated with myelin over the total signal, calculated as:

$$\textit{Specificity} = \frac{\textit{slope} * X_{\textit{max}}}{\textit{slope} * X_{\textit{max}} + Y_{\textit{intercept}}} \quad \textit{equation 2}$$

Where *slope* and *Y_intercept* are defined as the coefficients of the affine functions derived from the linear regression analyses. The *Y_intercept* is by definition the MRI signal measured in the absence of microscopy signal (i.e., for $X = 0$), and hence in the

1
2
3 absence of myelin. The product $slope * X_{max}$ represents the maximum MRI signal
4
5 corresponding to X_{max} , the maximum of microscopy signal (and hence the maximum
6
7 of myelin content). The product $slope * X_{max}$ thus corresponds to the fraction of ihMT
8
9 signal associated to myelin. The specificity, as defined here, is thus relative to a
10
11 reference anatomical structure, in this case, the internal capsules. Note that for the
12
13 specificity calculation, the $Y_intercept$ values were corrected for the offset
14
15 corresponding to the values of MR metrics in pure water: 0.3 Hz for R_1 (53) and 0.0%
16
17 for the MT-based metrics.
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25 *Sensitivity assessment*

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27
28 Sensitivities were evaluated for ihMTR and ihMTsat metrics, respectively based on the
29
30 slope values resulting from linear regression analyses with plp-GFP and LFB.
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33

34 *MPF vs. ihMT T_{1D} -filters*

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36
37 Pearson's correlations and linear regression analyses were performed between MPF
38
39 and ihMT T_{1D} -filters (ihMTR and ihMTsat) voxel-wise quantifications for a total of 51
40
41 common slices in the template space. Pairs of correlation coefficients and intercepts
42
43 for the ihMTR and ihMTsat configurations were tested for significant differences using
44
45 a Kruskal-Wallis test with post-hoc pairwise comparisons.
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49
50

51 **RESULTS**

52
53
54 MR templates for R_1 , MPF, ihMTR and ihMTsat at +0.7 mm, -1.0 mm, and -3.2 mm
55
56 from bregma are presented in Figure 2 along with the corresponding plp-GFP intensity
57
58 images. Qualitatively, R_1 showed a lower contrast between White Matter (WM) and
59
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2
3 Grey Matter (GM) tissues compared to ihMTR, ihMTsat and MPF. In addition, WM/GM
4 contrast was qualitatively improved in the ihMTsat images as compared to ihMTR.
5
6 Quantification of all metrics in each of the segmented structures are reported in
7
8 Supporting Information Table S1.
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12

13 *Plp-GFP vs. LFB*

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18 Linear regression and Pearson's correlation analyses between LFB and plp-GFP
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20 normalized signals (Figure 3) revealed a strong and significant correlation ($r^2 = 0.83$, p
21
22 < 0.001) with a positive significant slope (1.12 ± 0.12 , $p < 0.001$) and a non-significant
23
24 intercept (-0.16 ± 0.09 , $p = 0.11$).
25
26
27

28 *MRI vs. microscopy analyses*

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32 Outcomes of the linear regressions between the MR metrics and the plp-GFP and LFB
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34 signals are shown in Table 1, along with the corresponding scatter plots in Figure 4.
35
36 Residuals followed a normal distribution according to the Shapiro Wilk test ($p > 0.05$).
37
38 Strong and significant correlation coefficients were obtained for ihMT high-pass T_{1D} -
39
40 filters (ihMTR ($\Delta t_{0.0}$, $\Delta t_{0.8}$, $\Delta t_{3.2}$) and ihMTsat ($\Delta t_{0.0}$, $\Delta t_{0.8}$, $\Delta t_{3.2}$)) and MPF with regards
41
42 to plp-GFP ($r^2 \geq 0.79$, $p < 0.001$) and LFB ($r^2 \geq 0.87$, $p < 0.001$). Lower but significant
43
44 correlation coefficients corresponded to R_1 and the ihMT bandpass T_{1D} -filter.
45
46 Estimated Y_intercept values were generally significantly different than zero ($p < 0.05$),
47
48 except for the intercept of ihMTR ($\Delta t_{3.2}$) and ihMTsat ($\Delta t_{0.8}$, $\Delta t_{3.2}$) when compared to
49
50 plp-GFP. Globally, the specificity to myelination for the investigated MR metrics was
51
52 higher for associations with plp-GFP than for associations with LFB except for the ihMT
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3 bandpass T_{1D} -filter, for which similar specificity was found when plp-GFP or LFB was
4
5 used for association. The specificity of the ihMT high-pass T_{1D} -filters exhibited an
6
7 increasing trend with increasing Δt , while ihMTsat specificity values were superior to
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9 those of ihMTR for all Δt values.
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14 *Comparisons between the linear regression analyses: MR metrics as the common*
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16
17 *variable*
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21 No significant differences were found between pairs of correlation coefficients and
22
23 pairs of slopes when MR metrics were considered as the common variable, suggesting
24
25 similar sensitivities of each MR metric to the two microscopy references. However,
26
27 significant differences ($p < 0.05$) were found between pairs of intercept values for all
28
29 the MR metrics, except for the ihMT bandpass T_{1D} -filter and R_1 (Supporting Information
30
31 Table S2).
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37 *Comparisons between the linear regression analyses: microscopy metrics as the*
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39 *common variable*
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42
43 The significance of differences between pairs of overlapping correlation coefficients
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45 with either plp-GFP or LFB as the common variable are presented in Table 2.
46
47 Significant differences were generally found when comparing ihMT bandpass T_{1D} -filter
48
49 or R_1 correlation coefficients with ihMT high-pass T_{1D} -filters or MPF correlation
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51 coefficients. For ihMT metrics, the significance of differences between pairs of
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53 intercepts and pairs of slopes with either plp-GFP or LFB as the common variable are
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55 presented in Table 3. Significant differences were noticed for comparisons between all
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4 pairs of intercepts, except for comparisons between $\Delta t_{0.0}$ with $\Delta t_{0.0} - \Delta t_{0.8}$ (ihMTR and
5
6 ihMTsat) and $\Delta t_{0.8}$ with $\Delta t_{3.2}$ (ihMTR and ihMTsat) for associations with plp-GFP, and
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8 between $\Delta t_{0.8}$ and $\Delta t_{0.0} - \Delta t_{0.8}$ (ihMTR and ihMTsat) for associations with LFB.
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11 Differences between pairs of slopes were significantly different for all ihMTR and
12
13 ihMTsat metrics ($p < 0.05$), except for comparisons between ihMTR ($\Delta t_{0.0} - \Delta t_{0.8}$) and
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15 ihMTR ($\Delta t_{3.2}$) for associations with LFB or ihMTsat ($\Delta t_{0.0} - \Delta t_{0.8}$) with ihMTsat ($\Delta t_{3.2}$) for
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17 associations with plp-GFP and LFB.
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22 ***MPF vs. ihMT T_{1D} -filters analyses***

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26 Figure 5 and Figure 6 represent the distribution of ihMT high-pass and bandpass T_{1D} -
27
28 filters as compared to the distribution of MPF data points by means of 2D histograms
29
30 and illustrate the corresponding slices. The signal intensity distribution is different
31
32 between the various slices as a consequence of a varying number of voxels
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34 corresponding to WM (high signal intensity) or GM (low signal intensity) tissues. Here
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36 we can notice the importance of the ihMTsat metric in re-establishing the signal
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38 distribution in each slice. The effect is more pronounced for the bandpass T_{1D} -filter.
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45 Correlations of MPF with ihMT high-pass T_{1D} -filters (Figure 7a) were significant and
46
47 strong ($r^2 > 0.64$, $p < 0.001$ for ihMTR, and $r^2 > 0.72$, $p < 0.001$ for ihMTsat) for all
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49 slices. Significant ($p < 0.001$) but lower and more dispersed correlations were obtained
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51 for the ihMT bandpass T_{1D} -filter. All estimated slope and intercept values were
52
53 significantly different from zero ($p < 0.001$). Intercepts increased for increasing Δt , for
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55 ihMT high-pass T_{1D} -filters. Lowest intercept values were obtained for ihMT high-pass
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4 T_{1D} -filters with the lowest cutoff ($\Delta t_{0.0}$) and for the ihMT bandpass T_{1D} -filter (Figure 7b).

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6 Examples of linear regression analyses are given in Supporting Information, figures S1
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8 and S2.
9

10 11 DISCUSSION

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15 *Increasing the fraction of ihMT signal corresponding to long T_{1D} s enhances the*
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18 *specificity to myelination.*
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21 The assessment of the specificity was based on the evaluation of the intercept and
22
23 slope values resulting from linear regression analyses between the MR metrics and
24
25 the normalized signals of two microscopy references. In this approach, the intercept
26
27 and slope values are competitive estimates. For the specificity to increase with the
28
29 lengthening of Δt , the decrease in the intercept must be more important than the
30
31 decrease in the slope. According to the results in Part 1, this is due to the
32
33 disproportionate attenuation of long and short T_{1D} signals as Δt increases. High-pass
34
35 T_{1D} -filters increase the fraction of ihMT signal corresponding to long T_{1D} s and reduce
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37 that of short T_{1D} s (Part I, Table 2), leading to an enhanced specificity to myelination.
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45 The specificity dependence on Δt observed in this study for ihMT high-pass T_{1D} -filters
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47 is in agreement with previously reported results (41). However, specificity values
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49 corresponding to the plp-GFP reference obtained here are generally higher compared
50
51 to those reported by Duhamel et al. (41). While a discrepancy arising from a calibration
52
53 bias of the plp-GFP signal may not be excluded, several differences regarding the RF
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55 saturation parameters with a direct influence on the T_{1D} filtering mechanisms might
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3 explain the improvement in our results. The main advantage of this work consisted of
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5 the use of a modified version of ihMT (54) allowing us to maintain constant pulse power
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7 throughout all ihMT acquisitions. Thus, the sole effects of the switching time were
8
9 consistently isolated, and the specificity was more accurately assessed and compared
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11 across ihMT variants. Furthermore, partial volume effects were approximately 13 times
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13 lower per voxel in the current work, and the mouse cohort investigated here was larger.
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20 ***Short T_{1D} components are poorly correlated with myelin.***
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23 Since the intercept designates the value of a specific MR metric obtained for a null plp-
24
25 GFP or LFB signal, it actually relates to the part of the MR signal corresponding to a
26
27 non-myelin source. Interestingly, for associations with the plp-GFP signal, the $\Delta t_{0,0}$
28
29 configuration containing a high amount of signal originating from the short T_{1D} s (Part I,
30
31 Table 2) had a slightly higher, but not significantly different intercept as compared to
32
33 the band-pass T_{1D} -filter which mainly isolates the short T_{1D} s. As Δt increased, the
34
35 fraction of signal corresponding to the short T_{1D} s diminished as compared to the
36
37 fraction of signal corresponding to long T_{1D} s, and the intercept decreased as well. For
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39 example, the intercept of the $\Delta t_{3,2}$ configuration which had a higher signal contribution
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41 from the long T_{1D} s, was significantly different from the intercept of both of the $\Delta t_{0,0}$ and
42
43 $\Delta t_{0,0} - \Delta t_{0,8}$ configurations for associations with both plp-GFP and LFB signals. In
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45 addition, the r^2 correlation coefficient of the band-pass T_{1D} -filter was significantly lower
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47 than that of any of the high-pass T_{1D} -filters containing long T_{1D} s in various amounts.
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49 This allowed us to conclude that the non-myelin part of the ihMT signal is preferentially
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51 associated to the signal of short T_{1D} components.
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3 However, the source of the various T_{1D} s in in vivo tissues has still to be determined.

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6 While some short T_{1D} s could also be present in myelin tissue, another important
7
8 possible source could be the non-myelin macromolecules with a restrained movement,
9
10 presumably associated with glial cells. For example, the fibrous astrocytes with
11
12 elongated morphology and processes aligned with the myelinated fibers present in WM
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14 (55), are a potential candidate for the short T_{1D} component. Further dedicated studies
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16 will however be required to confirm the precise nature of short T_{1D} non-myelin
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18 molecules revealed by the ihMT bandpass T_{1D} -filter.
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25 ***IhMTsat improves the specificity of ihMT to myelination.***

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28 The correction for confounding B_1^+ inhomogeneity and R_1 relaxation effects in the ihMT
29
30 signal is vital for proper clinical studies, reproducibility, and multi-site comparisons.
31
32 Moreover, as shown in this study, the relative WM/GM contrast is qualitatively
33
34 enhanced for ihMTsat and the specificity to myelination is considerably improved.
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36 While various available approaches (48,56,57) demonstrated their utility, the ihMTsat
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38 approach presented by Munsch et al. (38) and implemented here has the advantage
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40 of a minimum number of assumptions.
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47 ***MR metrics specificity is higher for associations with plp-GFP, than with LFB.***

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50 The two microscopy references investigated in this study led to non-identical specificity
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52 values for MR metrics and generally higher values for associations with plp-GFP (7%
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54 higher for MPF and higher than 11% for ihMT high-pass T_{1D} -filters). For R_1 and the
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56 ihMT bandpass T_{1D} -filter the difference in the absolute specificity values calculated
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3 with either plp-GFP or LFB was inferior to 5%. Additionally, for these metrics, linear
4
5 regression analyses presented non-significant differences in the intercept and slope
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7 values for associations with plp-GFP and LFB, potentially suggesting a non-significant
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9 difference between specificities.
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14 The distinct underlying sources of signal for the two microscopy references may
15
16 explain the discrepancies between specificity values for MPF and ihMT high-pass T_{1D} -
17
18 filters. Lipids and proteins alike contribute to the MRI contrast (58,59), but disparate
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20 MT effects have been observed depending on the molecules. Increased MT effects
21
22 were obtained for cerebroside as a result of a higher number of exchangeable protons
23
24 and greater accessibility to free water (60). Conversely, phospholipids with fewer
25
26 exchangeable protons such as dimyristoylphosphatidylcholine (DMPC) and
27
28 phosphatidylcholine (PC) were found to have reduced MT effects (60,61). Although
29
30 such studies only considered a limited number of myelin components, the lower
31
32 specificity obtained for associations between the MT-derived metrics (ihMTR, ihMTsat,
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34 and MPF) and LFB might be explained by a lower sensitivity of MT effects to the
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36 presence of certain phospholipids, as opposed to the LFB quantifications (62,63). For
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38 example, PC is a major lipid of biological membranes accounting for 12% of the myelin
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40 lipids in rodents (64). In addition, phospholipids present in the membrane of other
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42 neural cells, such as astrocytes (65), are also stained by LFB, thereby reducing the
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44 specificity of LFB for the myelin membrane.
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56 On the other hand, transgenic mice expressing the green fluorescent protein driven by
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58 the proteolipid protein gene promoter have been developed to investigate cells in the
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3 oligodendrocyte lineage (66,67), offering a high specificity to mature oligodendrocytes
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6 in the adult brain (68) and thus a more specific biomarker for the myelin membrane.
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9 ***MPF is weighted toward short T_{1D} components.***

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12 MPF quantifications in the various ROIs are consistent with previously reported results
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14 using the single-point method (17). The extrapolated specificity of MPF calculated with
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16 the slope and intercept values reported by Khodanovich et al. (12) for associations with
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18 LFB on C57BL/6 healthy mice is higher by 15% as compared to the specificity in our
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20 study (66% vs. 51%). Both intercept and slope values are lower in our study. A lower
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22 number of data points for the linear regression analyses in this study combined with
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24 microscope calibration biases and a different staining and storage procedure of the
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26 brain slices might explain this difference.
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34 Generally, the lower specificity of MPF to myelination can be explained by the
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36 underlying sensitivity of the saturation efficacy in z-spectrum analyses. Although
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38 dipolar order effects were shown to have moderate to significant impact on the
39
40 accuracy of MT data fitting depending on the investigated tissue (9,69), the binary spin-
41
42 bath model used for MPF estimation does not consider any dipolar order contribution.
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44 This has particularly problematic consequences. Long RF pulses with low
45
46 instantaneous power as used in MT-prepared SPGR sequences are inefficient in
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48 creating dipolar order effects with regards to the short T_{1D} components. The saturation
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50 of the corresponding Zeeman pools is therefore not altered, and the MT effects of the
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52 short T_{1D} components are well modeled by the two-pool model. Conversely, for longer
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4 T_{1D} components, the RF pulses generate dipolar order effects, which alter the
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6 corresponding Zeeman pool saturation, but are not considered by the two-pool model.
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8 The MT effects of long T_{1D} components are thus not appropriately modeled. The
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10 estimated MPF derived from the classical binary spin-bath model is therefore
11
12 preferably weighted toward short T_{1D} components, associated with a low specificity to
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14 myelination.
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20 These observations are further confirmed by the voxel-wise regression analyses
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22 between MPF and ihMT. The lowest intercept values and thus the ihMT configurations
23
24 the closest to being in a direct proportionality relationship with MPF correspond to ihMT
25
26 ($\Delta t_{0.0}$), the signal of which is weighted by short T_{1D} components and ihMT ($\Delta t_{0.0} - \Delta t_{0.8}$),
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28 which specifically isolates short T_{1D} s.
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33 Thereupon, the effects of dipolar interactions on the MPF estimations in in vivo brain
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35 tissues and for the experimental scenarios used here should be considered in the
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37 future. Alternatively, cosine-modulated RF saturation pulses, which decouple Zeeman
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39 and dipolar order pools, should be employed for MPF estimations when using the
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41 classical binary-spin bath model.
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46 ***R_1 , MPF and ihMT: practical considerations for myelin imaging.***

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50 From a clinical point of view, the ideal technique for myelin imaging should be highly
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52 sensitive and specific, have a straightforward computation and relatively short
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54 acquisition time. None of the techniques investigated in this work meets all the criteria.
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4 In terms of sensitivity, R_1 , MPF, and to a lesser extent the $\Delta t_{0.0}$ ihMT configuration offer
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6 the best SNR performance and are also moderately to strongly correlated to
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8 myelination ($r^2 = 0.67$ for R_1 and > 0.8 for MPF and ihMT). Unfortunately, these are
9
10 also the least specific metrics due to a non-negligible contribution from non-myelin
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12 tissue to their signal, reflected by the high $Y_intercept$ values. Almost all the
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14 investigated MR metrics have non-zero $Y_intercept$, which is not surprising since
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16 macromolecules other than myelin contribute to T_1 relaxation, the MPF size, and the
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18 dipolar order exists in other macromolecules as well. An accurate estimation of each
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20 metric in non-myelin neuronal tissue would allow compensating for the non-zero
21
22 $Y_intercept$, but this is not an easy task and could be further complicated in the case
23
24 of pathology.
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33 In this context, a great advantage of ihMT over the other metrics is that there is no
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35 need to accurately quantify and correct for the signal of non-myelin neuronal tissue to
36
37 improve the specificity. A better myelin selectivity can be achieved through the
38
39 lengthening of Δt , and hence for specificity purpose, ihMT high-pass T_{1D} -filters ($\Delta t \geq 0.8$
40
41 ms) are very good candidates although they have lower SNR. The group standard
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43 deviation of ihMTR values in the seven investigated ROIs (Table S1) represents in
44
45 average less than 7% of the mean ihMTR value for $\Delta t_{0.0}$ but more than 17% for $\Delta t_{3.2}$.
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47 Lengthening Δt over 3.2 ms would probably induce a prohibitive SNR for accurate
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49 specificity measurements. Here, the minimum SNR obtained for $\Delta t_{3.2}$ (isolating mainly
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51 long T_{1Ds}) and the bandpass T_{1D} -filter (isolating mainly short T_{1Ds}) was high enough to
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3 accurately demonstrate a higher specificity of $\Delta t_{3.2}$ than that of the bandpass T_{1D} -filter
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6 by more than 1.5 standard errors.
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9 Regarding the computational simplicity (Figure 1), ihMTR is the only metric derived
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11 from a simple combination of the raw images. Other metrics require a more advanced
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13 knowledge of their respective model, hence complicating their derivation. In terms of
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15 acquisition time, ihMTR ($\Delta t < 3.2$ ms) and R_1 required less than 12 minutes, while
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17 ihMTR ($\Delta t_{3.2}$) and MPF required more than 15 minutes. These acquisition times might
18
19 appear too long from a clinical perspective, but they are in line with typical preclinical
20
21 acquisition times for parametric imaging with a 100 μm in-plane resolution. Moreover,
22
23 in clinical applications, the nominal resolution is lower ($\sim 1.5 - 2$ mm), and the degree
24
25 of myelination is higher (leading to higher ihMTR values), thus the ihMT high-pass T_{1D}
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27 filters strategies are routinely feasible ($\Delta t = 0.0$ ms (47) and $\Delta t > 0.0$ ms (35)).
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36 To summarize, the higher sensitivity of R_1 , MPF and the $\Delta t_{0.0}$ high-pass T_{1D} -filter
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38 (ihMTR/ihMTsat) can be advantageously used for the general characterization of
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40 diseases for which myelin alterations are part of the pathological processes. On the
41
42 other hand, high-pass T_{1D} -filters with long Δt values should be preferred to investigate
43
44 whether myelin is specifically altered in pathologies.
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49 *Limitations*

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53 First, an important limitation in the assessment of the specificity in the current study
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55 was the lower spatial resolution of the MR slices (750 μm) as compared to that of the
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57 microscopy slices (20 μm). To partially overcome this issue, three slices of 20 μm were
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3 sampled inside the corresponding MR slice thickness of 750 μm and the resulting
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5 signals were averaged. Second, the specificity as defined here is not an absolute
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7 measure, but rather a relative estimation referenced to the signal of a known highly
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9 myelinated structure (internal capsules). Normalization to another structure with a
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11 higher degree of myelination may yield slightly different specificity values. Finally, for
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13 a more comprehensive qualitative and quantitative analysis on the specificity of
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15 different MR metrics, future studies should comprise a larger number of microscopy
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17 markers including antibodies for other myelin specific proteins, lipid dyes or even more
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19 advanced techniques such as Coherent Anti-Stokes Raman Spectroscopy (CARS)
20
21 (70) which can be tuned to the specific vibrational frequency of methylene groups
22
23 present along the lipid chains. Comparison with other MR metrics putatively sensitive
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25 to myelin but whose contrast rely on different mechanisms, e.g., Myelin Water Fraction,
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27 should also be performed (71).
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37 38 CONCLUSIONS

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41 The specificity to myelination of ihMT high-pass and bandpass T_{1D} -filters, MPF and R_1
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43 was assessed using two histological reference techniques for myelin imaging.
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45 Lengthening of the Δt leads to an increase in the fraction of signal corresponding to
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47 long T_{1D} s and thus provided higher specificities to myelination for ihMT high-pass T_{1D} -
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49 filters, albeit at the cost of lower sensitivity. The correction for T_1/B_1 effects allowed us
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51 to further enhance the specificity of ihMT to myelination and generate images with
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53 higher WM/GM contrast.
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3 The ihMT bandpass T_{1D} -filter, which isolates the short T_{1D} components (< 1 ms) whose
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5 nature will require future dedicated studies, provided lower specificity values.
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8 Additionally, an important contribution of short T_{1D} components to the MPF estimation
9
10 can explain the lower specificity of MPF to myelination as compared to the ihMT high-
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12 pass T_{1D} -filters.
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17 Future studies with ihMT T_{1D} -filters would be of particular interest when analyzing ihMT
18
19 signal variations in pathological brain conditions. As T_{1D} is sensitive to membrane
20
21 organization (31), the myelin structural transformation occurring in some diseases
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23 (e.g., loss of the normal compact multilamellar structure observed in MS (72)) might
24
25 induce changes on the short and long T_{1D} components, and hence modify the ihMT
26
27 signal in the brain. Thus, ihMT bandpass and high-pass T_{1D} -filters with properly
28
29 adjusted T_{1D} cutoff values could determine the ranges of T_{1D} components affected by
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31 the pathological processes.
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44
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Figure Captions

Main document

Table 1: Results of linear regression analyses between the MR metrics with the two microscopy references (MR signal = Y_intercept + Normalized plp-GFP Signal * slope and MR signal = Y_intercept + Normalized LFB Signal * slope). The values of the Y-intercept, slope and specificity are presented as estimated value \pm standard error. Coefficients significantly different than zero (p -value < 0.05) are represented by the asterisk symbol (*). The specificity estimation of R_1 (\dagger), for which the Y_intercept was corrected with the value of the metric in pure water measured at room temperature (53) might be slightly overestimated due to the higher in vivo temperature.

Table 2: Results of comparisons between pairs of overlapping correlation coefficients resulted from associations between the MR metrics with the two microscopy references. Plp-GFP (top) and LFB (bottom) are considered as the common variable. Significant p -values (p -value < 0.001 and p -value < 0.05) are accompanied by the asterisk symbol (*) and the 95% confidence intervals are reported. Pairs not indicated in this table were not significantly different.

Table 3: Results of comparisons between pairs of slopes (top) and intercepts (bottom) for ihMTsat (left) and ihMTR (right) when either plp-GFP (above the main diagonal) and LFB (below the main diagonal) was considered as the common variable.

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Significant p-values (p-value < 0.001 and p-value < 0.05) are accompanied by the asterisk symbol (*).

Figure 1: Diagram summarizing the acquisition and processing steps from the raw images to the final quantitative and semi-quantitative metrics investigated in this study. The acquisition time for each MR sequence was: 4 mins 32 s for qMT; 9 mins 30 s for VFA; 1 min 59 s for AFI and 1 min 40 s for ihMTR. In the context of this study, the acquisition time necessary for the derivation of each metric was: 11 mins 29 s for R_1 , 16 mins 01s for MPF, 8 mins 40 s for ihMTR ($\Delta t_{0,0}$) and 20 mins 9s for ihMTsat ($\Delta t_{0,0}$), 11 mins 20 s for ihMTR ($\Delta t_{0,8}$) and 22 mins 49 s for ihMTsat ($\Delta t_{0,8}$), 22 mins for ihMTR ($\Delta t_{3,2}$) and 33 mins 29 s for ihMTsat ($\Delta t_{3,2}$). The increasing time of ihMTR/ihMTsat as Δt is lengthened is due to an increasing number of averages of the raw MTw images. IhMTR and B_1 maps were generated by direct computation, whereas R_1 , ihMTsat and MPF maps were generated via model fitting.

Figure 2: Templates of MR metrics and the corresponding fluorescence images at three levels of bregma (+0.7 mm, -1.0 mm, and -3.2 mm from bregma). The seven segmented ROIs are indicated by red arrows on the Plp-GFP and the T_2 -weighted images.

Figure 3: Linear regression analysis between the LFB and plp-GFP normalized signals. One data point corresponds to the ROI average of one structure from one mouse.

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3 Figure 4: Linear regression analyses between the MR metrics with the plp-GFP
4 normalized signal (a - d) and the LFB normalized signal (e-h). One data point
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9 corresponds to the ROI average of one structure from one mouse.

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12 Figure 5: Bivariate histograms for MPF and ihMTR $\Delta t_{0.0}$ (a, f, k), MPF and ihMTR $\Delta t_{0.8}$
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14 (b, g, l), MPF and ihMTR $\Delta t_{3.2}$ (c, h, m), MPF and $\Delta t_{0.0}-\Delta t_{0.8}$ (d, i, n) for three different
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17 slices from the caudal to the rostral part of the brain (e, j, o). The bins were calculated
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20 with the Freedman–Diaconis rule.

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23 Figure 6: Bivariate histograms for MPF and ihMTsat $\Delta t_{0.0}$ (a, f, k), MPF and ihMTsat
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25 $\Delta t_{0.8}$ (b, g, l), MPF and ihMTsat $\Delta t_{3.2}$ (c, h, m), MPF and $\Delta t_{0.0}-\Delta t_{0.8}$ (d, i, n) for three
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28 different slices from the caudal to the rostral part of the brain (e, j, o). The bins were
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31 calculated with the Freedman–Diaconis rule.

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34 Figure 7: Plots of (a) r^2 correlation coefficients and (b) intercepts for the linear
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37 regression analyses between MPF and ihMT metrics on the 51 common template
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40 slices. The mean value of the distribution of each coefficient in all investigated slices
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43 is represented by a black line, and the median value by a red line. The wider sections
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46 of the violin plot represent a higher probability of the coefficients to take on the
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49 corresponding values represented on the x-axis. All the tested pairs were significantly
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52 different (p -value < 0.05), except for the comparisons accompanied by the
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55 corresponding non-significant p -values.

56 *Supporting Information*
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4 Supporting Information Table S1: MR and microscopy quantifications in seven CNS
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6 structures (mCC – medial corpus callosum, INT – internal capsules and OPT – optical
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8 tract, TH – thalamus, CP – caudoputamen, CTX- cerebral cortex, HIP – hippocampal
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10 region). Mean absolute values and group standard deviations were calculated on the
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12 ROI averages of the six plp-GFP mice.
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17 Supporting Information Table S2: Results of comparisons between pairs of correlation
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19 coefficients, slope, and Y_intercept estimated values resulted from associations of
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21 each MR metric with the two microscopy references. MR metrics are considered as
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23 the common variable. Significant p-values (p-value < 0.05) are accompanied by the
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25 asterisk symbol.
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31 Supporting Information Figure S1: Example of linear regression analyses between
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33 MPF and ihMTR voxel-wise quantifications (MPF signal = Y_intercept + ihMTR signal
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35 * slope) on six different slices from the caudal to the rostral part of the brain (a to f).
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37 The corresponding $\Delta t_{0,0}$, $\Delta t_{0,8}$, MPF, and $\Delta t_{0,0} - \Delta t_{0,8}$ images in the signal ranges [0%,
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39 23%], [0%, 15%], [0%, 10%], and [0%, 10%] respectively, are illustrated.
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45 Supporting Information Figure S2: Example of linear regression analyses between
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47 MPF and ihMTsat voxel-wise quantifications (MPF signal = Y_intercept + ihMTsat
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49 signal * slope) on six different slices from the caudal to the rostral part of the brain (a
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51 to f). The corresponding $\Delta t_{0,0}$, $\Delta t_{0,8}$, MPF, and $\Delta t_{0,0} - \Delta t_{0,8}$ images in the signal ranges
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53 [0%, 23%], [0%, 15%], [0%, 10%], and [0%, 10%] respectively, are illustrated.
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Table 1: Results of linear regression analyses between the MR metrics with the two microscopy references (MR signal = Y_intercept + Normalized plp-GFP Signal * slope and MR signal = Y_intercept + Normalized LFB Signal * slope). The values of the Y-intercept, slope and specificity are presented as estimated value ± standard error. Coefficients significantly different than zero (p-value < 0.05) are represented by the asterisk symbol (*). The specificity estimation of R₁ (†), for which the Y_intercept was corrected with the value of the metric in pure water measured at room temperature (53) might be slightly overestimated due to the higher in vivo temperature.

MR metrics	Normalized plp-GFP Signal				Normalized LFB Signal				
	r ²	Y_intercept	Slope (%)	Specificity (%)	r ²	Y_intercept	Slope (%)	Specificity (%)	
R ₁	* 0.67	* 0.4 ± 0.02	* 18.6 ± 2.4	† 65 ± 5	* 0.77	* 0.4 ± 0.01	* 16.0 ± 2.1	† 62 ± 4	
MPF	* 0.83	* 3.8 ± 0.3	* 5.1 ± 0.4	58 ± 7	* 0.91	* 4.8 ± 0.2	* 4.4 ± 0.3	51 ± 5	
ihMTR	Δt _{0.0}	* 0.85	* 5.7 ± 0.9	* 14.5 ± 1.2	73 ± 10	* 0.91	* 8.5 ± 0.7	* 12.6 ± 1.0	62 ± 7
	Δt _{0.8}	* 0.86	* 1.7 ± 0.6	* 10.9 ± 0.8	87 ± 12	* 0.88	* 4.3 ± 0.6	* 8.8 ± 0.8	70 ± 10
	Δt _{3.2}	* 0.79	0.3 ± 0.4	* 5.7 ± 0.5	95 ± 17	* 0.88	* 1.5 ± 0.3	* 5.0 ± 0.4	79 ± 11
	Δt _{0.0} - Δt _{0.8}	* 0.46	* 4.0 ± 0.6	* 3.6 ± 0.7	49 ± 13	* 0.59	* 4.2 ± 0.5	* 3.8 ± 0.7	50 ± 12
ihMTsat	Δt _{0.0}	* 0.82	* 0.5 ± 0.2	* 3.3 ± 0.3	87 ± 14	* 0.91	* 1.1 ± 0.2	* 3.0 ± 0.2	75 ± 8
	Δt _{0.8}	* 0.84	0.04 ± 0.1	* 2.3 ± 0.2	98 ± 17	* 0.87	* 0.6 ± 0.1	* 1.9 ± 0.2	79 ± 13
	Δt _{3.2}	* 0.79	-0.04 ± 0.08	* 1.1 ± 0.1	104 ± 20	* 0.89	* 0.2 ± 0.06	* 1.0 ± 0.1	87 ± 15
	Δt _{0.0} - Δt _{0.8}	* 0.60	* 0.5 ± 0.1	* 1.1 ± 0.2	71 ± 20	* 0.76	* 0.6 ± 0.1	* 1.1 ± 0.1	68 ± 10

Table 2: Results of comparisons between pairs of overlapping correlation coefficients resulted from associations between the MR metrics with the two microscopy references. Plp-GFP (top) and LFB (bottom) are considered as the common variable. Significant p-values (p-value < 0.001 and p-value < 0.05) are accompanied by the asterisk symbol (*) and the 95% confidence intervals are reported. Pairs not indicated in this table were not significantly different.

plp-GFP as the common variable								
	ihMTsat			ihMTR			$\Delta t_{0.0} - \Delta t_{0.8}$	MPF
	$\Delta t_{0.0}$	$\Delta t_{0.8}$	$\Delta t_{3.2}$	$\Delta t_{0.0}$	$\Delta t_{0.8}$	$\Delta t_{3.2}$		
ihMTsat	* < 0.001	* 0.006	* 0.031	* < 0.001	* 0.002	* 0.031	* 0.004	* 0.004
($\Delta t_{0.0} - \Delta t_{0.8}$)	[0.06, 0.3]	[0.04, 0.3]	[0.01, 0.3]	[0.08, 0.3]	[0.05, 0.3]	[0.01, 0.3]	[-0.3, -0.02]	[-0.3, -0.05]
ihMTR	* < 0.001	* < 0.001	* 0.005	* < 0.001	* < 0.001	* 0.004		* < 0.001
($\Delta t_{0.0} - \Delta t_{0.8}$)	[-0.5, -0.1]	[-0.5, -0.09]	[-0.5, -0.06]	[0.13, 0.48]	[0.1, 0.5]	[0.06, 0.5]		[-0.5, -0.01]
R_1	> 0.05	* 0.047 [0.001, 0.2]	> 0.05	* 0.029 [0.01, 0.2]	* 0.019 [0.01, 0.3]	> 0.05		* 0.045 [0.003, 0.2]

LFB as the common variable

	ihMTsat			ihMTR			MPF	
	$\Delta t_{0.0}$	$\Delta t_{0.8}$	$\Delta t_{3.2}$	$\Delta t_{0.0}$	$\Delta t_{0.8}$	$\Delta t_{3.2}$		$\Delta t_{0.0} - \Delta t_{0.8}$
ihMTsat ($\Delta t_{0.0} - \Delta t_{0.8}$)	* 0.007 [0.03, 0.2]	> 0.05		* 0.008 [0.03, 0.2]	> 0.05		* < 0.001 [-0.3, -0.04]	* 0.022 [-0.2, -0.01]
ihMTR ($\Delta t_{0.0} - \Delta t_{0.8}$)	* < 0.001 [-0.4, -0.07]	* 0.017 [-0.4, -0.03]	* 0.007 [-0.4, -0.04]	* < 0.001 [0.08, 0.4]	* 0.016 [0.03, 0.4]	* 0.010 [0.04, 0.4]		* < 0.001 [-0.4, -0.05]
R_1	* 0.037 [0.005, 0.2]			> 0.05				* 0.037 [0.005, 0.2]

Table 3: Results of comparisons between pairs of slopes (top) and intercepts (bottom) for ihMTsat (left) and ihMTR (right) when either plp-GFP (above the main diagonal) and LFB (below the main diagonal) was considered as the common variable. Significant p-values (p-value < 0.001 and p-value < 0.05) are accompanied by the asterisk symbol (*).

Slope	ihMTsat				ihMTR			
	$\Delta t_{0.0}$	$\Delta t_{0.8}$	$\Delta t_{3.2}$	$\Delta t_{0.0} - \Delta t_{0.8}$	$\Delta t_{0.0}$	$\Delta t_{0.8}$	$\Delta t_{3.2}$	$\Delta t_{0.0} - \Delta t_{0.8}$
$\Delta t_{0.0}$		* 0.003	* < 0.001	* < 0.001		* 0.015	* < 0.001	* < 0.001

$\Delta t_{0.8}$	* 0.003		* < 0.001	* < 0.001	* 0.004		* < 0.001	* < 0.001
$\Delta t_{3.2}$	* < 0.001	* < 0.001		0.880	* < 0.001	* < 0.001		* 0.029
$\Delta t_{0.0} - \Delta t_{0.8}$	* < 0.001	* 0.001	0.540		* < 0.001	* < 0.001	0.180	
Intercept	ihMTsat				ihMTR			
	$\Delta t_{0.0}$	$\Delta t_{0.8}$	$\Delta t_{3.2}$	$\Delta t_{0.0} - \Delta t_{0.8}$	$\Delta t_{0.0}$	$\Delta t_{0.8}$	$\Delta t_{3.2}$	$\Delta t_{0.0} - \Delta t_{0.8}$
$\Delta t_{0.0}$		0.091	* 0.028	0.860		* < 0.001	* < 0.001	0.110
$\Delta t_{0.8}$	* 0.004		0.620	* 0.035	* < 0.001		0.071	* 0.012
$\Delta t_{3.2}$	* < 0.001	* 0.005		* 0.002	* < 0.001	* < 0.001		* < 0.001
$\Delta t_{0.0} - \Delta t_{0.8}$	* 0.004	0.880	* 0.001		* < 0.001	0.910	* < 0.001	

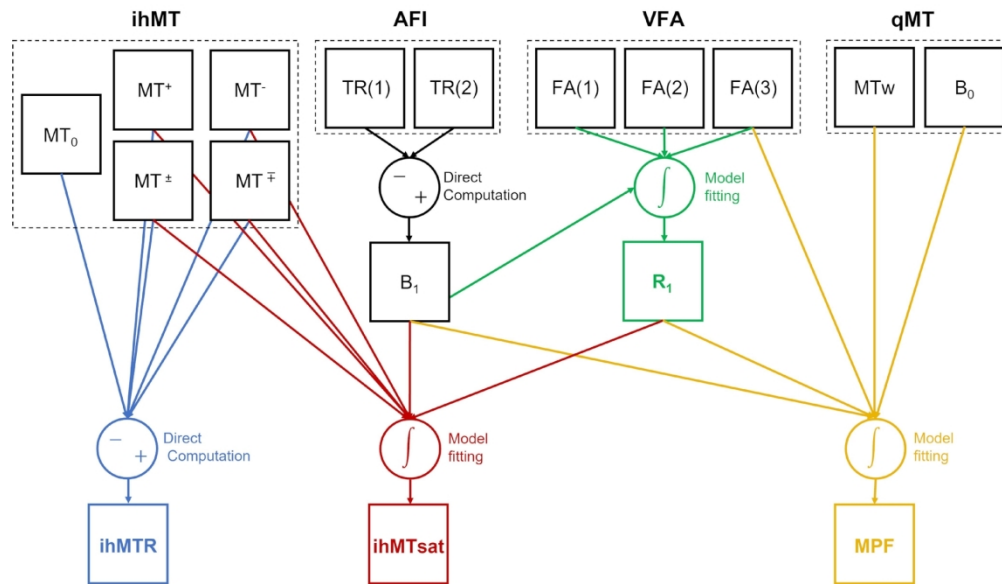


Figure 1: Diagram summarizing the acquisition and processing steps from the raw images to the final quantitative and semi-quantitative metrics investigated in this study. The acquisition time for each MR sequence was: 4 mins 32 s for qMT; 9 mins 30 s for VFA; 1 min 59 s for AFI and 1 min 40 s for ihMTR. In the context of this study, the acquisition time necessary for the derivation of each metric was: 11 mins 29 s for R_1 , 16 mins 01 s for MPF, 8 mins 40 s for ihMTR ($\Delta t_{0.0}$) and 20 mins 9 s for ihMTsat ($\Delta t_{0.0}$), 11 mins 20 s for ihMTR ($\Delta t_{0.8}$) and 22 mins 49 s for ihMTsat ($\Delta t_{0.8}$), 22 mins for ihMTR ($\Delta t_{3.2}$) and 33 mins 29 s for ihMTsat ($\Delta t_{3.2}$). The increasing time of ihMTR/ihMTsat as Δt is lengthened is due to an increasing number of averages of the raw MT_w images. IhMTR and B_1 maps were generated by direct computation, whereas R_1 , ihMTsat and MPF maps were generated via model fitting.

175x102mm (300 x 300 DPI)

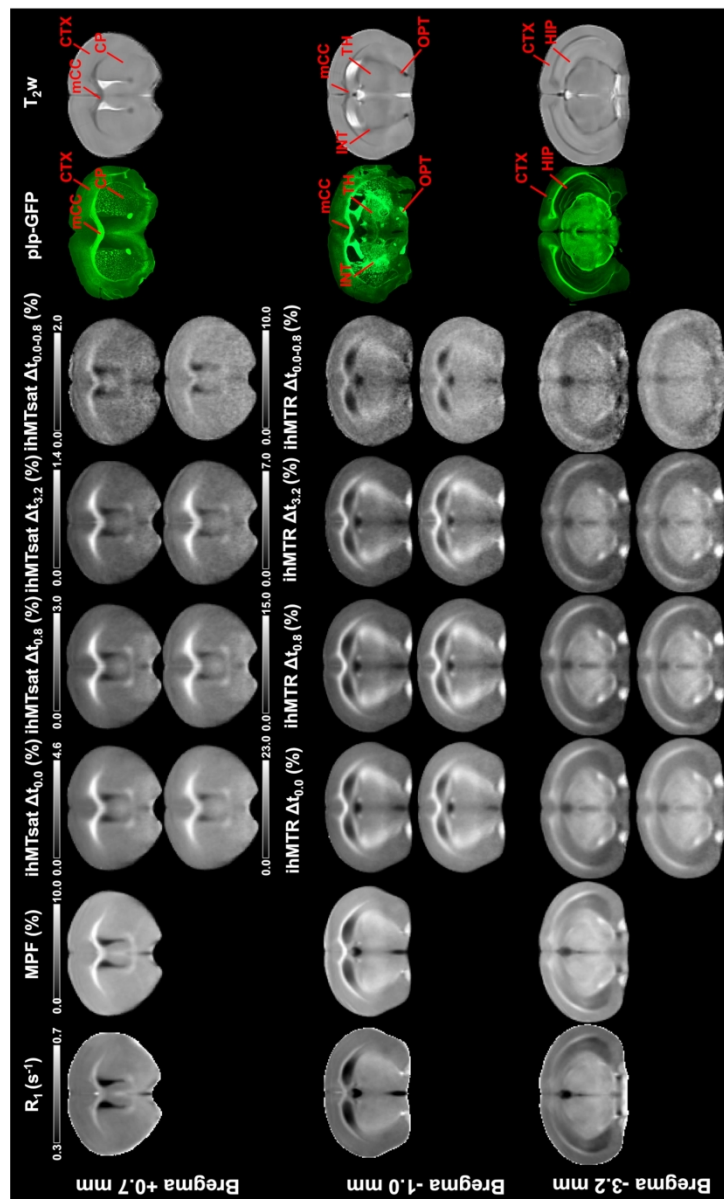


Figure 2: Templates of MR metrics and the corresponding fluorescence images at three levels of bregma (+0.7 mm, -1.0 mm, and -3.2 mm from bregma). The seven segmented ROIs are indicated by red arrows on the plp-GFP and the T₂-weighted images.

146x241mm (600 x 600 DPI)

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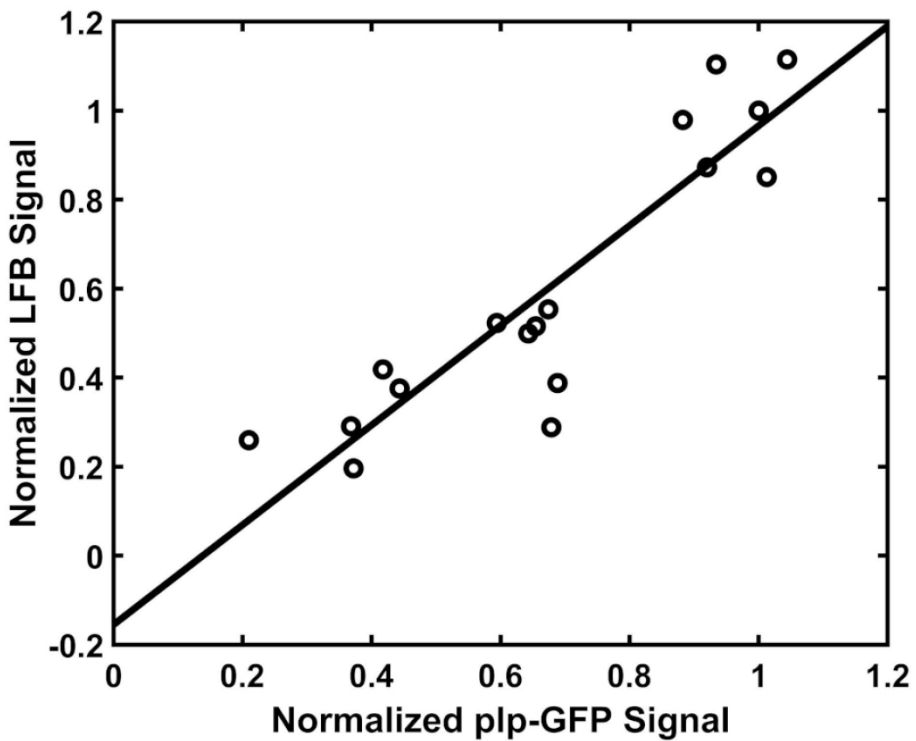


Figure 3: Linear regression analysis between the LFB and plp-GFP normalized signals.

86x67mm (600 x 600 DPI)

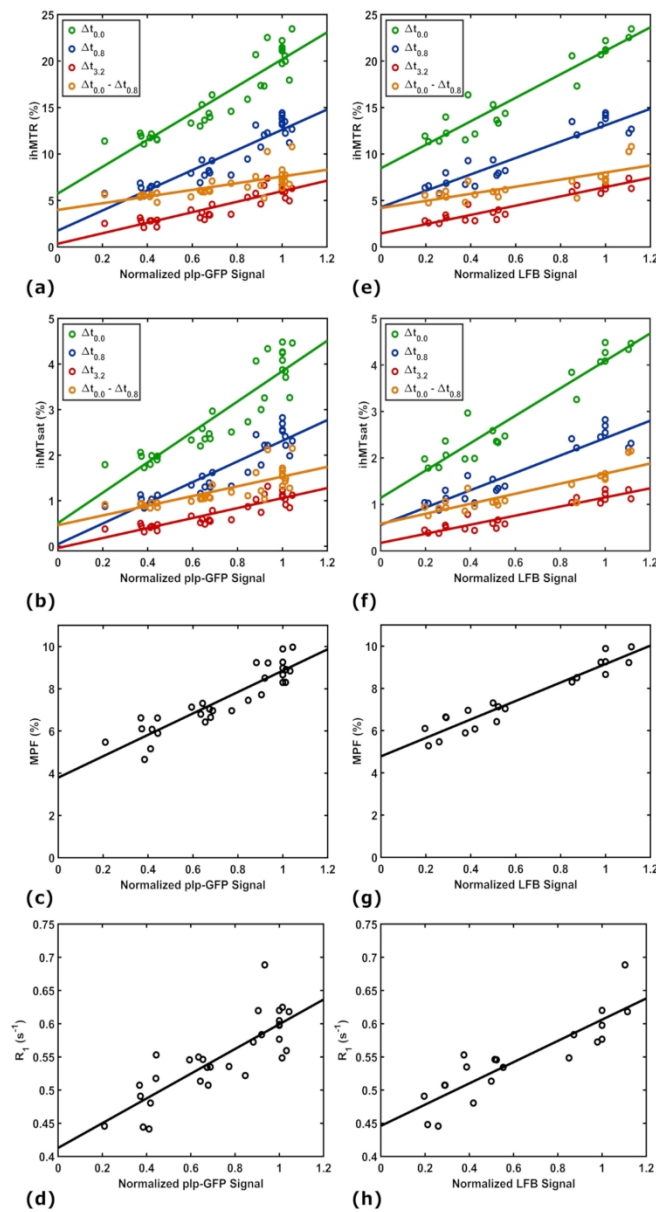


Figure 4: Linear regression analyses between the MR metrics with the plp-GFP normalized signal (a - d) and the LFB normalized signal (e-h). One data point corresponds to the ROI average of one structure from one mouse.

130x241mm (300 x 300 DPI)

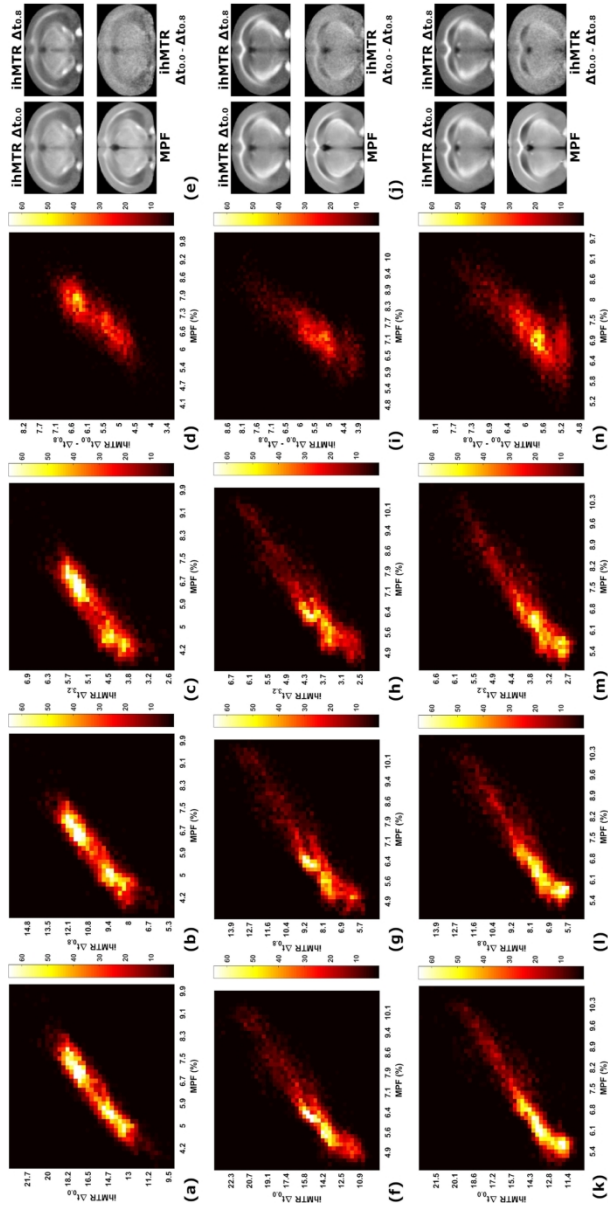


Figure 5: Bivariate histograms for MPF and ihMTR $\Delta t_{0,0}$ (a, f, k), MPF and ihMTR $\Delta t_{0,8}$ (b, g, l), MPF and ihMTR $\Delta t_{3,2}$ (c, h, m), MPF and $\Delta t_{0,0}-\Delta t_{0,8}$ (d, i, n) for three different slices from the caudal to the rostral part of the brain (e, j, o). The bins were calculated with the Freedman–Diaconis rule.

120x240mm (300 x 300 DPI)

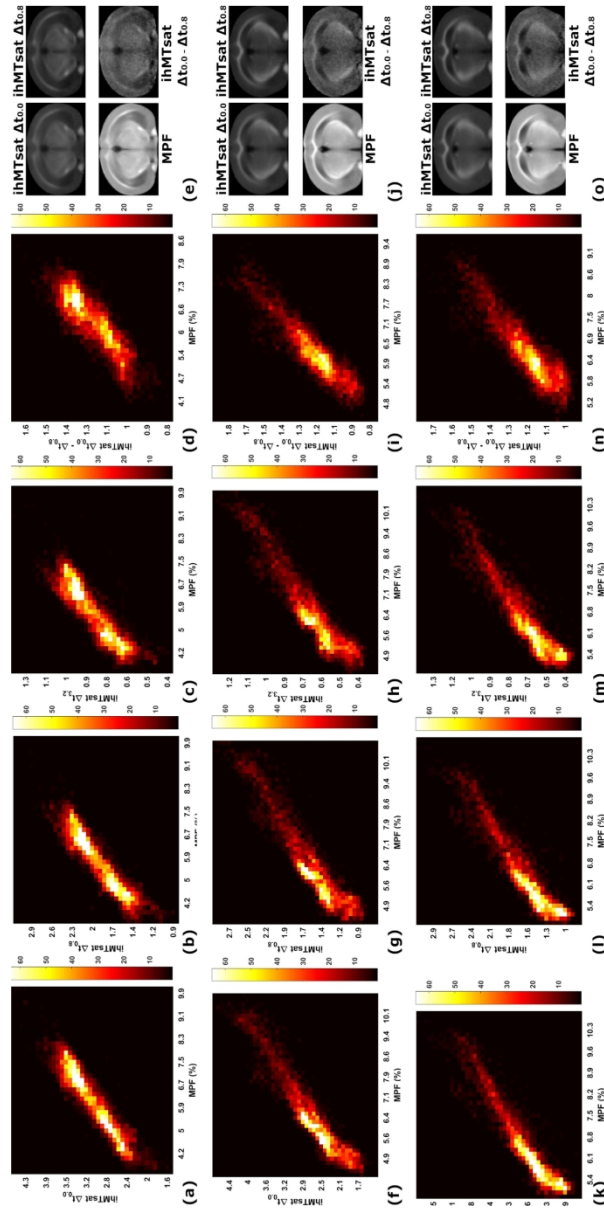


Figure 6: Bivariate histograms for MPF and ihMTsat $\Delta t_{0,0}$ (a, f, k), MPF and ihMTsat $\Delta t_{0,8}$ (b, g, l), MPF and ihMTsat $\Delta t_{3,2}$ (c, h, m), MPF and $\Delta t_{0,0}-\Delta t_{0,8}$ (d, i, n) for three different slices from the caudal to the rostral part of the brain (e, j, o). The bins were calculated with the Freedman–Diaconis rule.

119x240mm (300 x 300 DPI)

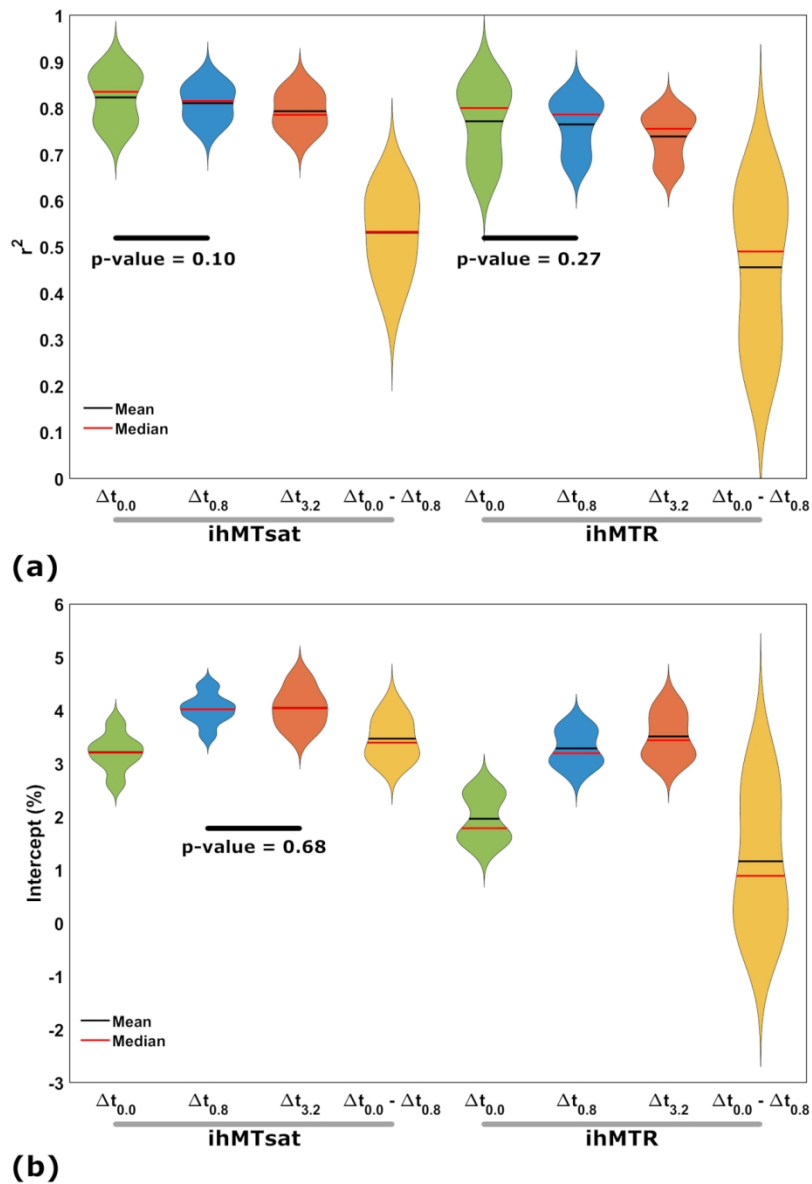


Figure 7: Plots of (a) r^2 correlation coefficients and (b) intercepts for the linear regression analyses between voxel-wise MPF and ihMT metrics on the 51 common template slices. The mean value of the distribution of each coefficient in all investigated slices is represented by a black line, and the median value by a red line.

The wider sections of the violin plot (73) represent a higher probability of the coefficients to take on the corresponding values represented on the x-axis. All the tested pairs were significantly different ($p < 0.05$), except for the comparisons accompanied by the corresponding non-significant p-values.

165x241mm (600 x 600 DPI)